Explosive Residue Detection Using Polypeptide-Based Biosensors

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The overall goal of the proposal was to create polypeptide-based biosensors that are targeted against 2,4,6-trinitrotoluene (TNT) or its breakdown products. To identify these receptors we developed an in vitro evolution and selection (IVES) process that leads to a greatly accelerated cycle of receptor modification and diversification. During this funding period we 1) substantially optimized the IVES process by increasing the sensitivity and reducing the false positive rate of the assay; 2) developed a signal-generation system that uses fluorescence resonance energy transfer for the detection of small organic compounds; and 3) identified a TNT receptor that specifically binds TNT compared to its metabolite DNT. The EC50 for the TNT receptor in the yeast-based one-hybrid assay is ~ 3 µM (0.7 ppm). The amount of TNT present in soil samples above a TM-62P mine buried 4” below the surface has been measured to be ~ 2 ppm. Thus, theoretically, a 2 ppm concentration of TNT should be detected by the TNT receptor that we developed through the IVES process.
Statement of the Problem Studied

The overall goal of the proposal is to create polypeptide-based biosensors that are targeted against the ligands, 2,4,6-trinitrotoluene (TNT) or its breakdown products, and which produce a fluorescent readout upon ligand binding. To identify polypeptide-based receptors that bind ligands that are of interest to the DoD we have developed an in vitro evolution and selection (IVES) process that leads to a greatly accelerated cycle of receptor modification and diversification. The IVES process uses the estrogen receptor (ER) as the ancestral receptor from which novel polypeptide-based receptors are derived. A yeast-based one-hybrid screen is used to identify receptors that bind the ligand of interest. The signal-generation system is based on the conformational change that occurs upon interaction of the polypeptide-based biosensors with a ligand.

Summary of the Most Important Results

Key Research Accomplishments:

- Optimized the yeast-based one-hybrid screen to identify novel polypeptide-based receptors.
- Developed a FRET-based signal-generation system for the detection of ligands.
- Identified a novel binding-pocket within the wild type ER ligand-binding domain (LBD) that binds small organic molecules, such as the pesticide, endosulfan-S.
- Identified a polypeptide-based receptor that selectively binds TNT compared to its metabolite 2-amino-4,6-dinitrotoluene (DNT). The EC50 for this TNT receptor in the yeast one-hybrid assay is ~ 3 µM (0.7 ppm).

Optimizing the Yeast-Based One-Hybrid Screen for the Detection of Novel Polypeptide-Based Receptors:

Substantial effort has been devoted to improving the signal-to-noise ratio of the yeast-based one-hybrid screening method (1). In the originally developed method the growth of the yeast was dependent on ligand-binding by the wild type ER (Fig. 1 and Fig. 2A). In the absence of ligand the ligand-binding domain (LBD) of the ER acts as a transcriptional repressor of the activation domain function 1 (AF1), which is located in the N-terminus of the ER (NER). In response to ligand-binding the LBD no longer represses transcription, which results in the expression of an enzyme that permits histidine synthesis. Thus the yeast are able to grow in media that does not contain histidine (H-). To create novel polypeptide-based receptors mutations are incorporated into the LBD. These receptors are then screened in the one-hybrid system to identify mutants that bind TNT or its metabolites. However, the introduction of mutations into the LBD results in both amino acid substitutions and stop codons. The introduction of stop codons generates truncated LBDs and the loss of ligand-dependent control over AF1 function. These constitutively active receptors are responsible for the high level of false positives observed during the screening process.

Figure 1 Schematic of the yeast-based one-hybrid method. In the absence of ligand the yeast are unable to grow in selection media without histidine. In the presence of ligand (eg, estradiol, (E)) the ER undergoes a conformation change that enhances gene expression and permits growth in selection media.
In an effort to overcome the high false positive rate we incorporated in frame at the C-terminal end of the estrogen receptor the cDNA for the blasticidin resistance protein (BSD) (Fig. 2B). Yeast grown in the presence of the antibiotic, blasticidin can only survive if they have expressed the full-length protein. Mutations that introduce truncations into the receptor prevent the expression of BSD. Therefore, yeast containing truncated receptors are killed in the presence of blasticidin, which should result in a reduction of the false positive level in the screening method. However, we found that the introduction of the BSD substantially reduced the transcriptional activity of the AF1 region and only the binding of high affinity ligands could be detected. It is likely that the “first-generation” of receptors will have a weak affinity for the ligand of interest and thus we reasoned that we would be unable to detect these receptors in this system.

In another approach to lower the number of false positives we generated a series of constructs in which we switched the position of the NER and the LBD to generate a GAL4DBD-LBD-NER fusion protein (Fig. 2C and 2D). We reasoned that the introduction of a stop codon in the LBD would produce a transcriptionally inactive protein because the NER would not be expressed. Furthermore, we determined whether we could increase the sensitivity of the screen by using the VP16 transcriptional activation function (VP16) instead of the AF1 region of the ER (Fig. 2E and 2F). The VP16 transcriptional activation function is a much more potent activator of transcription than AF1. Unfortunately, the transcriptional activity of VP16-TAF could not be controlled in a ligand-dependent manner. However, we found that the GAL4DBD-LBD-NER fusion protein showed dramatically enhanced transcriptional activity compared to that of GAL4DBD-NER-LBD. The improved signal-to-noise ratio of the new construct is illustrated using the LBD of the methoxychlor receptor (LBD(MC)) that we had previously identified using the IVES method. In the presence of methoxychlor (10 µM) only a few yeast colonies are observed with the GAL4DBD-NER-LBD(MC) construct whereas hundreds of colonies are observed using the GAL4DBD-LBD(MC)-NER construct (Fig. 3). No colonies were observed for either construct in the absence of methoxychlor. Furthermore, the transfection efficiencies were approximately similar for each construct (data not shown). It is not clear why switching the domains has such a dramatic effect on the transcriptional activity of the AF1 region. One possible explanation is that the overall protein folding of the NER differs between the fusion proteins and that these structural differences alter the transcriptional activity of the NER. Nonetheless, the fact that we dramatically increased the signal-to-noise level using the GAL4DBD-LBD-NER greatly improves the use of the yeast-based one-hybrid screen to identify novel receptors.

**Figure 2 Schematic of constructs.** See text for details.

**Figure 3 Switching the position of the LBD and NER dramatically increases the sensitivity of the yeast-based one-hybrid screen.** Yeast (Hf7C) were transfected with a plasmid encoding GAL4DBD-NER-LBD(MC) or GAL4DBD-LBD(MC)-NER. The transfected yeast were plated onto selection media (W-H-) in the presence or absence of 10 µM methoxychlor(MC). The transfection efficiencies for the two constructs were similar (data not shown). Yeast colonies appear as white circles. The heterogeneous sizes of the colonies are a characteristic feature of Hf7C.
In addition to improving the sensitivity of the screen we also identified the reason we have not been able to successfully isolate plasmids encoding mutant receptors from previous screens. The usual approach in the one-hybrid screen is to isolate the plasmid encoding the mutant receptor from the yeast and then transform bacteria with the plasmid. Sufficient quantities of the plasmid can be obtained from bacteria so that the sequence of the mutant receptor can be identified. This approach worked well for identifying methoxychlor receptors. However, we could not successfully rescue the plasmid containing the mutant receptor in screens with TNT or its metabolite, DNT. For reasons not clear to us the cDNA for the mutant receptor as well as the gene conferring tryptophan resistance is stably integrated into the yeast genome during the screens with TNT and DNT. We are now able to isolate the mutant receptors by amplifying the mutant receptor sequence. The amplified product is then directly sequenced.

Isolation and Identification of a TNT receptor:

**Figure 4** Characterization of the ligand specificity of the TNT receptor. Yeast (Hf7c) were transfected with GAL4DBD-LBD(TNT)-NER (LBD(TNT)) or GAL4DBD-LBD(WT)-NER. Aliquots of the transfected plates were spread on selection plates without tryptophan and histidine (W-H-) with or without 10 µM TNT, DNT, endosulfan-S (EDS), dieldrin (DLD) and toxaphene (TXP). Aliquots plated on the W- plates were used to determine transfection efficiency.

In previous screening attempts with TNT using the prototype construct we identified a number of extremely weak TNT receptors. However, now that we had dramatically enhanced the sensitivity of our assay by using the GAL4DBD-LBD-NER construct, we tested whether these TNT receptors would give a more robust response in the improved assay. The LBD of the various TNT receptors were subcloned into GAL4DBD-LBD-NER, and these constructs were transformed into yeast. We found that one of the TNT receptors supported the growth of yeast in the presence but not in the absence of TNT (Fig. 4). The specificity of this response is shown by the inability of the TNT receptor to permit the growth of yeast in 10 µM DNT. The construct containing the wild type LBD (LBD(WT)) did not permit the growth of yeast in TNT or DNT. This result was not due to differences in transfection efficiency between the constructs as the number of colonies on selection plates without tryptophan (W-) was actually greater for the construct encoding LBD(WT) compared to LBD (TNT)(Fig. 4). Tryptophan is used as a selection marker for the plasmid.

We further characterized the ligand binding specificity of the TNT receptor and found that it did not permit the growth of yeast in the presence of the organochlorine pesticides, dieldrin or toxaphene (Fig. 4). However, both the LBD(WT) and LBD(TNT) supported the growth of yeast in the presence of 10 µM endosulfan-S, an organochlorine pesticide. Interestingly, endosulfan-S does not directly compete with estradiol for binding (2). These results suggest that an endosulfan binding-pocket distinct from that of estradiol exists in the ER. We constructed various N- and C-terminal deletion mutants of the
LBD(WT) and found that the endosulfan binding-pocket is located within residues 301 to 425 in the full-length WT human ER. We also determined that a transcriptional activation function was present between residues 301 to 359. This transcriptional activation function, referred to as AF2a, was previously identified to be between residues 282 to 351 of the human ER (3). Therefore, we were able to map the N-terminal location of AF2a more precisely.

The LBD(TNT) was sequenced and was found to contain a frame shift, which would be located at amino acid 425 in the full-length wild type human ER. The frame shift results in the introduction of a stop codon ~11 amino acid residues from the frame shift and therefore, the remainder of the LBD and NER are not translated. Thus the TNT receptor activates transcription through AF2a and TNT interacts with the endosulfan-binding pocket. However, at this point we have not determined whether the 11 amino acids that are divergent between the TNT receptor and the wild type ER are important for TNT binding. Nonetheless, we have identified the first receptor that is able to switch on gene expression in response to the presence of TNT. This “first-generation” receptor has an EC\textsubscript{50} in the yeast one-hybrid assay of ~3 \textmu M (0.7 ppm) (Fig. 5). The variability of the response is most likely due to differences in expression levels of the TNT receptor between the independent colonies that were analyzed. Expression levels will be significantly influenced by the location of integration of the cDNA for the TNT receptor. TNT found around landmines is largely due to leakage from within the mine. The amount of TNT present in soil samples above a TM-62P mine buried 4” below the surface has been measured to be ~2 ppm. Thus this concentration should be detected using our “first-generation” TNT receptor. We anticipate that further mutagenesis and screening would allow us to identify a “second generation” TNT receptor that would demonstrate an improved affinity and specificity compared to the original TNT receptor.

**Figure 5** Dose response of TNT receptor. Three independent yeast colonies that had been transfected with GAL4DBD-LBD(TNT)-NER were inoculated into selection media in the presence of varying concentrations of TNT. The colonies were grown in suspension and turbidity was measured 48 hr after inoculation.

**Signal-Generation System:**

The development of the signal-generation system is described in full in the attached manuscript, which was submitted to Journal of Steroid Biochemistry and Molecular Biology. In brief, we developed a prototype signal-generation system that uses fluorescence resonance energy transfer for the detection of small organic molecules (4).

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**List of all Publications and Technical Reports**

(a) Papers published in peer-reviewed journals – None

(b) Papers published in non-peer-reviewed journals or in conference proceedings – De, S., Macara, I.G. and Lannigan, D.A. Polypeptide-based biosensors for chemical sensing. (2004) ARO workshop for on-chip detection of biological and chemical molecules.

(c) Papers presented at meetings, but not published in conference proceedings – None


(e) Technical reports submitted to ARO – Lannigan, D.A. (Interim and Final Progress Reports) Explosive Residue Detection Using Polypeptide-Based Biosensors
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Report of Inventions


Bibliography


